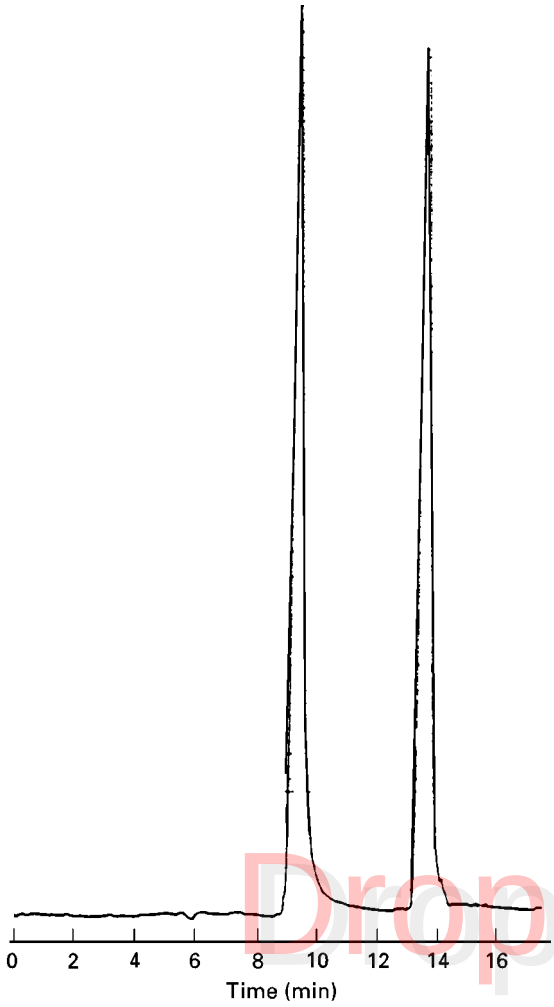


**Figure 7** Electrochromatographic resolution of methylphenyl barbitone by a Chiralcel OJ (Daicel, Japan) chiral stationary phase. The cellulose ester derivative commercially coated on to silica was packed into a 30 cm (21 cm packed and 22 cm to detection window) × 75 μm i.d. fused silica capillary. The mobile phase was acetonitrile –water (70 : 30 v/v), applied voltage 15 kV and temperature 25°C.

shorter overall migration times. Many different areas are showing considerable research interest, such as electrochromatography, where in the future commercially available columns may prevail over in house methods of packing capillaries with chiral stationary phases. Generally, commercial interest in the area of capillary packings is likely to increase as more 'tailor-made' phases are introduced.

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# Cellulose and Cellulose Derived Phases

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## Introduction

The properties of cellulose derivatives for the separation of enantiomers were recognized in 1966 by Lütringhaus and Peters. The full potential of microcrystalline cellulose triacetate (CTA) was definitely established by Hesse and Hagel in 1973. Meanwhile, microcrystalline cellulose tribenzoate became commercially available. Another milestone in the development of cellulose- and amylose-based phases was the work of Okamoto and co-workers who in 1984 introduced polysaccharide derivatives coated on a macroporous silica. Currently such materials are certainly the most universally applicable type of commercially available chiral stationary phases. Then, in 1988 Francotte and co-workers introduced cellulose esters of aromatic acids in the form of almost spherical, partially crystalline particles with a relatively

good mechanical stability. The last few years have also seen a lot of research in grafting cellulose and amylose derivatives on to a silica matrix.

Recently, different types of polysaccharide phase (Chiralcel AD, Chiralcel AS, Chiralcel OD and Chiralcel OJ) wall-coated open tubular (WCOT) columns have also been used for supercritical fluid chromatography applications.

Here, the properties and possibilities of different types of polysaccharide phases used for enantiomer separation are discussed.

## Microcrystalline Cellulose Triacetate

By a heterogeneous acetylation procedure, cellulose can be acetylated so that the mutual arrangement of the carbohydrate chains within their crystalline regions is maintained. CTA obtained by this acetylation technique has only weak chiral recognition abilities. Only in its swollen state is triacetyl cellulose capable of separating enantiomers. An ethanol–water (95 : 5, v/v) mixture is the most frequently used swelling agent for CTA. When dry CTA is boiled for 15 min in the ethanol–water mixture, a volume increase of about 40% is observed.

Microcrystalline CTA is a material that can be used to separate a broad variety of enantiomers belonging to many different classes of organic compounds (thiazoline-2 thiones, barbiturates, hydrocarbons, oxiranes, flavanones, $\gamma$- and $\delta$-lactones, metal complexes, etc.) Its high loading capacity makes it an extremely useful material for preparative chromatographic purposes.

### Mobile-Phase Design

A lot of different solvents, e.g. acetone, chlorinated alkanes and acetonitrile, cannot be used because they dissolve CTA more or less completely. In tetrahydrofyran, CTA forms a gel. In some other solvents, like for example, 1,4 dioxane and dimethoxyethane, CTA swells too strongly. Different investigators found as a general rule that the retention factors of the investigated analytes increased when, respectively, methanol, ethanol or propanol was used as the eluent. However, the highest selectivity factors are often found with ethanol as the eluent. Successful separations are also obtained by using other low molecular weight alcohols, ethers, hydrocarbons and mixtures of these eluents. Examples are methanol-2-propanol (80 : 20; v/v); ethanol–*tert*-butyl methyl-ether–water (86 : 10 : 4, v/v) and *n*-hexane–2-propanol–water (70 : 27 : 3, v/v).

In general, a significant influence on the retention factor and enantioselectivity is observed when ethanol as the eluent is modified with methanol, 2-propanol, different amounts of water, or is completely replaced by methanol or 2-propanol.

If compounds bearing an ionizable group have to be analysed, buffer systems should be tried. CTA seems to withstand the use of buffers in the range between pH 5 and 10 for a long time without any significant loss of chromatographic properties. Eluents with a high water content at high or low pH values should be avoided because CTA can be hydrolysed under these conditions.

Empirically, it has been found that, on CTA successful enantiomer separations can be expected if the analytes possess an aromatic or nonaromatic ring close to the chiral centre or if the products have an asymmetric atom on a rigid ring structure.

Also it is known from experience that compounds bearing an ionizable group, like for example, a hydroxyl, carboxyl or amino group, are generally poorly resolved on CTA.

Derivatization of these functional groups into the corresponding ester, amide or carbamate derivative often improves the selectivity. For alcohol, an esterification reaction with a *P*-substituted benzoic acid chloride (F, Cl, Br, CN, $NO_2$, $OCH_3$) has been successfully applied to solve a number of different separation problems.

CTA is attractive for chiral separations because it can be synthesized from a low cost natural product. The wide range of products that can be separated, together with its high loading capacity, certainly makes it a material that should be considered as a valuable tool in the development of chiral separation methods. Besides the usefulness of CTA in the field of enantiomer separations, this material is also suitable for the separation of positional isomers.

## Microcrystalline Cellulose Tribenzoate

As early as 1969, Safanova and Klenkova had described the heterogeneous benzoylation of microcrystalline cellulose. Rimböck *et al*. later described a method for preparing this material using an ultrasonic field to accelerate the reaction. Nowadays, a methanol–water solution containing about 40% of the pre-swollen material is commercially available.

Comparable with CTA, different solvents or solvent combinations also cause different degrees of swelling of this material. Dichloromethane or tetrahydrofuran cannot be used as the eluent because tribenzoylcellulose is soluble in these solvents.

With microcrystalline cellulose tribenzoate, a stationary phase for chromatographic enantiomer separations has been introduced, which allows the resolving of different classes of organic compounds.

Cellulose tribenzoate, compared to some other types of chiral stationary phases, offers the advantage that, besides analytical separations, preparative work can also be performed without a great deal of effort.

## Benzoyl Cellulose Beads (in the Pure Polymeric Form)

Francotte and co-workers developed this type of chiral stationary phase. These materials are cellulose esters of aromatic acids in the form of almost spherical, partially crystalline particles with a good mechanical stability.

To characterize these phases, a broad variety of product classes on experimental batches of benzoyl- and *p*-methylbenzoyl cellulose beads have been examined. Methanol, ethanol and *n*-hexane-2-propanol were used as the eluent and the results obtained were compared with data measured under the same experimental conditions on the corresponding physically coated materials (Chiralcel OB and Chiralcel OJ).

In general, much higher retention values are observed on the pure cellulose derivatives compared to the values measured on the corresponding coated stationary phases. This observation is logical, because the physically coated phases only contain about 20 weight percent of the cellulose derivative. For the investigated product series, in most cases smaller retention values were measured for methanol than for ethanol as the mobile phase. Furthermore, most products also demonstrated the highest resolution values when methanol was used as the eluent.

For some of the substances tested, practically no difference in resolution was observed between the pure cellulose material and the coated phases, while other product classes were always better separated on the commercially available columns.

### Mobile-Phase Effects

A chromatogram of the Mesuximid enantiomer separation that clearly illustrates the separation power of this type of materials is depicted in **Figure 1**.

For Mesuximid, using *n*-hexane–2 propanol as the mobile phase, the investigation of the effect of the polar modifier concentration on the retention factor demonstrate that, below a certain polar modifier concentration, the retention factor strongly increases. This can be an indication that competition between the polar modifier and the solute for hydrogen-binding sites on the stationary phase might be the determining factor in the retention process.

Although the retention factor is strongly influenced by the polar modifier concentration, the effect on the selectivity factor in the studied range of polar modifier content remains rather small.

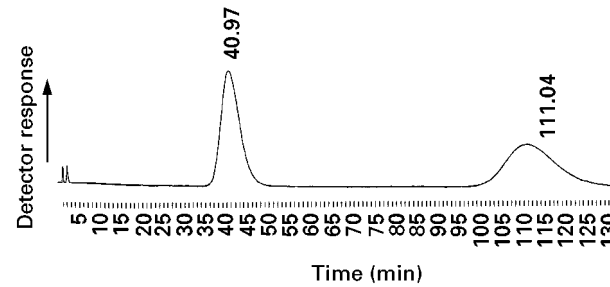


**Figure 1** Benzoyl cellulose beads in the pure polymeric form: effect of polar modifier concentration on the retention factor. Column: 125 × 4 mm i.d. filled with 10 μm *p*-methylbenzoyl cellulose beads. Mobile phase: *n*-hexane–2-propanol in different ratios. Flow rate: 1 mL $min^{-1}$. Sample: Mesuximid.

The benzoyl cellulose derivatives in the pure polymeric form allow the separation of a broad variety of compounds. In many cases, results are obtained which are comparable with the values measured on the corresponding physically coated phases. Furthermore, these materials are very useful as stationary phases for preparative chromatographic applications.

## Physically Coated Cellulose and Amylose Derivatives

This type of chiral stationary phase, developed by Okamoto and commercialized by Daicel Chemical Industries, can be considered to be the most universally applicable type of chiral stationary phase for both analytical and preparative chromatographic applications. Many different column types are available on the market. However, experience suggests that, with four different types of phases, a wide range of racemates, belonging to a broad variety of product classes, can be separated. The most interesting phases in our view are:

1. Cellulose derivatives:
   (a) Chiralcel OD (3,5 dimethylphenyl carbamate derivative)
   (b) Chiralcel OJ (*para*-methylbenzoyl derivative)
2. Amylose derivatives:
   (a) Chiralpak AD (3,5 dimethylphenyl carbamate derivative)
   (b) Chiralpak AS [(*S*)-α-methylbenzyl carbamate derivative)]

The analytical phases are coated on a 10 μm wide pore silica. However, some types of phases are also available on a 5 μm silica matrix. Furthermore, two phases, which are specifically designed for reversed-phase applications, have been brought on to the market. An overview of the different types of commercially available coated phases is given in **Table 1**.

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**Table 1** Commercially available silica-coated phases for enantioseparation

| *Cellulose esters* | *Type of absorbent* |
|---|---|
| Chiralcel OJ | *p*-Methylbenzoyl |
| Chiralcel OJ-R | For reversed-phase application |
| Chiralcel OB | Benzoyl |
| Chiralcel OB-H | Coated on a 5 μm silica matrix |
| Chiralcel OA | Acetyl |
| Chiralcel OK | Cinnamoyl |
| Chiralcel CA-1 | Acetyl |
| *Cellulose carbamates* | |
| Chiralcel OD | 3,5-Dimethylphenyl |
| Chiralcel OD-H | Coated on a 5 μm silica matrix |
| Chiralcel OD-R | For reversed-phase applications |
| Chiralcel OD-RH | For reversed-phase applications |
| Chiralcel OC | Phenyl |
| Chiralcel OG | *p*-Methylphenyl |
| Chiralcel OF | *p*-Chlorophyenyl |
| *Amylose carbamates* | |
| Chiralpak AD | 3,5 Dimethylphenyl |
| Chiralpak AS | (*S*)α-Methylbenzyl |

## Mobile-Phase Design and Parameter Optimization

Different investigators have suggested that the mechanism of chiral recognition on cellulose and amylose derivatives is based on:

1. Hydrogen bonding
2. Dipole–dipole interaction
3. Charge transfer complex formation (π–π interactions)
4. Possible inclusion into chiral cavities or channels of the chiral stationary phase

Whatever the type of interaction involved, the mobile phase must be considered as a dynamic part of the system, capable of interacting with both the enantiomeric solute and the chiral stationary phase. For solutes where hydrogen bonding plays an important role in the selective chiral interaction process, proton-donating polar modifiers can compete with the solute for the hydrogen-bonding sites of the stationary phase. In other cases, π–π interactions between an aromatic moiety on the solute and the chiral stationary phase seems to be the most important interaction force.

Most of the separations on physically coated cellulose and amylose columns have been performed with mobile phases consisting of *n*-hexane or *n*-heptane as the major component, mixed with various aliphatic alcohols. The choice of solvent combination is principally based on recommendations for use by the manufacturer. Concerns about the stability of these columns combined with the relatively high cost discouraged the use of other solvent combinations than those recommended by the manufacturer. However, in the last few years, other aprotic solvents, e.g., acetonitrile, methyl-tertiary-butyl ether and ethyl acetate have been applied on certain types of the derivatized polysaccharide columns. Certainly in the field of preparative chromatographic enantiomer separations. These alternative solvents widen the application range of this type of stationary phase.

For method development on this type of phase screening experiments on $50 \times 4.6$ mm ID pre-columns filled with respectively Chiralcel OJ, Chiralcel OD, Chiralpak AD and Chiralpak AS are convenient. In use such column are rapidly equilibrated, give fast separations and have in many cases more than sufficient separation power for initial method development work. As a general rule, pure ethanol at a flow rate of 0.5 mL $min^{-1}$ is used as the eluent of choice to perform the first experiments.

Pure ethanol is chosen based on the experience that, for a lot of products, below a certain amount of polar modifier in an *n*-hexane or *n*-heptane based mobile phase, the retention factors strongly increase. In general, the effect of the polar modifier on the retention factor decreases upon increasing modifier content. An indication that the competition between the solute and the polar modifier in the eluent for the hydrogen-bonding sites of the stationary phase is a saturable process and a maximum effect on the retention factor will be reached within a certain range of polar modifier concentration (for our type of compounds, mostly situated between 15 and 20%). If, in the screening experiments, the analytes are insufficiently retained or no separation is observed, ethanol is mixed with *n*-heptane or *n*-hexane in different ratios. Once a suitable *n*-heptane–ethanol ratio has been found to reach a $k'$ value between 3 and 6, ethanol is replaced by the same molar amount of one of the other lower aliphatic alcohols (1- or 2-propanol, primary, secondary or tertiary butanol). In many cases, large effects on the resolution values can be observed when ethanol as polar modifier is replaced by another alcohol.

Experience shows that pure methanol or mixtures of ethanol and methanol or ethanol and 2-propanol are often very useful to improve a separation.

On these phases it has been observed that the retention factors of a range of 2,3-dihydro-1,4-benzodioxin-2-carboxylic acid esters increased with increased chain length of the alcohol used for chromatography on a Chiracel OB column. This effect might be based on a reduced capability of the larger alcohols to compete with the solute for hydrogen-binding sites on the stationary phase.

The higher retention values measured for the branched alcohols compared to their corresponding linear analogues may be due to steric influences, which result in a reduced tendency of these alcohols to interact by hydrogen bonding with the polysaccharide phase.

The observed decrease in retention factor with increasing chain length of the ester group could be an indication that hydrophobic effects also contribute to the interaction mechanism between the solute and the chiral stationary phase.

For the lower members of the homologue series of the 2,3-dihydro-1,4-benzodioxin-2-carboxylic acid esters, practically no difference in resolution values can be observed between the different polar modifiers. Depending on the type of alcohol used, the resolution rapidly decreases for the pentyl ester and the higher homologues. Only for *n*-butanol as polar modifier, is the decrease in resolution value rather limited. For ethanol the lowest resolution values are observed for the whole product series. There are indications that, for the investigated solutes, hydrogen-bonding forces certainly play an important role in the chiral recognition process.

This type of experiment clearly demonstrates that, when *n*-hexane or *n*-heptane–alcohol mixtures are used, testing different alcohols as polar modifier during method development should be performed, because in many cases, large differences in enantioselectivity may be observed.

It is also interesting to note that, by changing the polar modifier phase, an inversion of the elution order can occur, as illustrated in the following example. On a Chiralcel OD column, it was possible to separate the investigated compound (R89439) in its two enantiomers using a mixture of *n*-hexane–ethanol in an 80 : 20 volume ratio. The desired enantiomer eluted as the first peak under these experimental conditions, as illustrated in **Figure 2**A. When the ethanol as polar modifier was partially replaced by methanol, a reversal of elution order was observed, as illustrated in **Figure 2**B.

Some investigators have suggested that binding a polar modifier to sites near the chiral cavities might alter the steric environment of these cavities. If the environment of these cavities changes, it can certainly have an influence on the steric fit of the chiral solutes in these cavities, which may in part be responsible for the observed phenomenon of reversal in elution order. It is furthermore interesting to note that a reversal of elution order can often be achieved by the changeover of a carbamate-type phase towards a benzoate-type phase. This knowledge can be very useful when a small amount of one enantiomer has to be determined besides a large excess of the other enantiomer, because it is easier to quantify a small peak in front of a large one than in the opposite situation. Based on experience, it is also important to mention that sometimes even small variations in experimental conditions can have a tremendous effect on the resolution of enantiomers on this type of stationary phases.

The often observed strong influence of small differences in experimental conditions on the resolution, certainly has to be considered when robust and reproducible methods have to be developed on this type of phases, for regulatory (Good Laboratory (GLP), Good Manufacturing (GMP)) purposes.

An example of the use of aprotic modifiers, which can also affect the separation on these phases, is as follows. About 40 g of a diastereomeric mixture had to be separated into its four enantiomers. Very good separation was obtained on an amylose 3,5-dimethylphenyl carbamate (Chiralpak AD) column using pure ethanol, pure methanol or a mixture of ethanol and 2-propanol in a 90 : 10 volume ratio. Unfortunately, the solubility of the diastereomeric mixture in the alcohol used was very poor. We therefore decided to study the behaviour of ethanol–acetonitrile mixtures, because the solubility of the product was a lot better in acetonitrile.

The results of these experiments are summarized in **Table 2** and graphically represented in **Figure 3**.

As illustrated in **Figure 3**, the addition of an aprotic modifier to the polar organic solvent has a clear effect on the retention behaviour and the enantioselectivity, especially for the most retained enantiomers. The investigated solute molecule contains different hydrogen-bonding groups, which can strongly interact with the carbamate functionality of the stationary phase. Combined with poor solubility of the substance in the lower molecular weight alcohols, this makes these solvents less suitable to displace the solute from the stationary phase and probably explains the high retention values observed for pure methanol or ethanol.

The addition of acetonitrile improves the solubility of the product. This at first simplifies the transfer of the solute into the mobile phase and furthermore gives the protic solvent a better chance to compete for the hydrogen-bonding sides of the stationary phase, which may explain the observed systematic decrease in retention factors with increasing acetonitrile concentration. Below a certain concentration of protic solvent in the eluent, the strong hydrogen-bonding properties of the solute predominate again, resulting in an increase in retention factor. Using a mixture of ethanol and acetonitrile in a 30 : 70 volume ratio allowed the separation of the diastereomeric mixture in its four enantiomers without difficulty.

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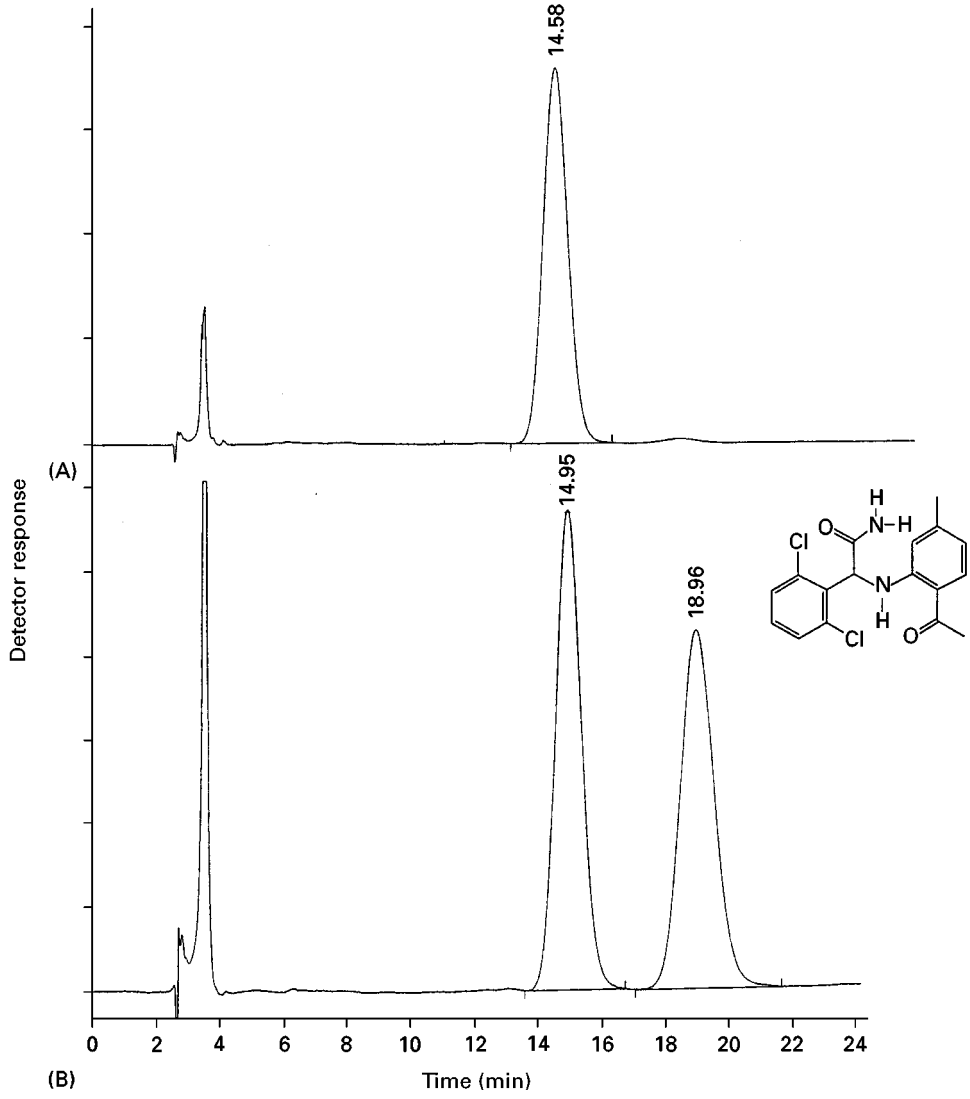


**Figure 2** Effect of mobile-phase composition on the elution order. Column: 250 × 4.6 mm. Chiralcel OD (cellulose 3,5 dimethylphenyl carbamate). Mobile phase: (A) *n*-hexane–ethanol (80 : 20; v/v); (B) *n*-hexane–ethanol–methanol (80 : 5 : 15; v/v). Flow rate: 1 mL $min^{-1}$. Sample: R89439 (racemate plus pure enantiomer).

**Table 2** Effect of the addition of acetonitrile on retention behaviour and enatioselectivity

| *Acetonitrile (%)* | *k′1* | *k′2* | *k′3* | *k′4* | *α (1–2)* | *α (3–4)* |
|---|---|---|---|---|---|---|
| 0 | 2.94 | 6.43 | 11.97 | 28.24 | 2.187 | 2.359 |
| 5 | 1.80 | 3.6 | 8.67 | 14.30 | 2 | 1.649 |
| 10 | 1.25 | 2.7 | 7.55 | 10.25 | 2.16 | 1.358 |
| 15 | 0.95 | 2.08 | 6.34 | 8.03 | 2.189 | 1.267 |
| 20 | 0.66 | 1.59 | 5.47 | 6.62 | 2.409 | 1.210 |
| 30 | 0.50 | 1.25 | 5.26 | 5.26 | 2.50 | 1.0 |
| 40 | 0.46 | 1.1 | 5.05 | 5.05 | 2.391 | 1.0 |
| 50 | 0.45 | 1.04 | 5.72 | 5.72 | 2.311 | 1.0 |
| 70 | 0.90 | 1.73 | 9.38 | 12.05 | 1.922 | 1.285 |
| 80 | 1.31 | 2.67 | 16.86 | 27.0 | 2.038 | 1.601 |

Basic or acidic additives can also have major effects on the separations. Thus, for some basic or acidic substances, it is often necessary to add respectively a base or an acid to improve the peak shape. Based on the manufacturer's recommendations, it is possible to use up to 1% of di- or triethylamine to reduce the tailing of basic substances. Acetic or trifluoroacetic acid can be used for the analysis of acidic substances.

The usefulness of these mobile-phase additives, is shown in the following example. For the enantiomer separation of [( ± ) 2,6-dichloro-α-(4-chlorophenyl)-4-(4,5-dihydro-3,5-dioxo-1,2,4-triazin-2-(3H)-yl) benzene acetonitrile] (diclazuril) initially a method was used in which the acidic NH-group in the 3,5-dioxo-1,2,4 triazin part of the molecule was methylated with diazomethane. The derivatized product

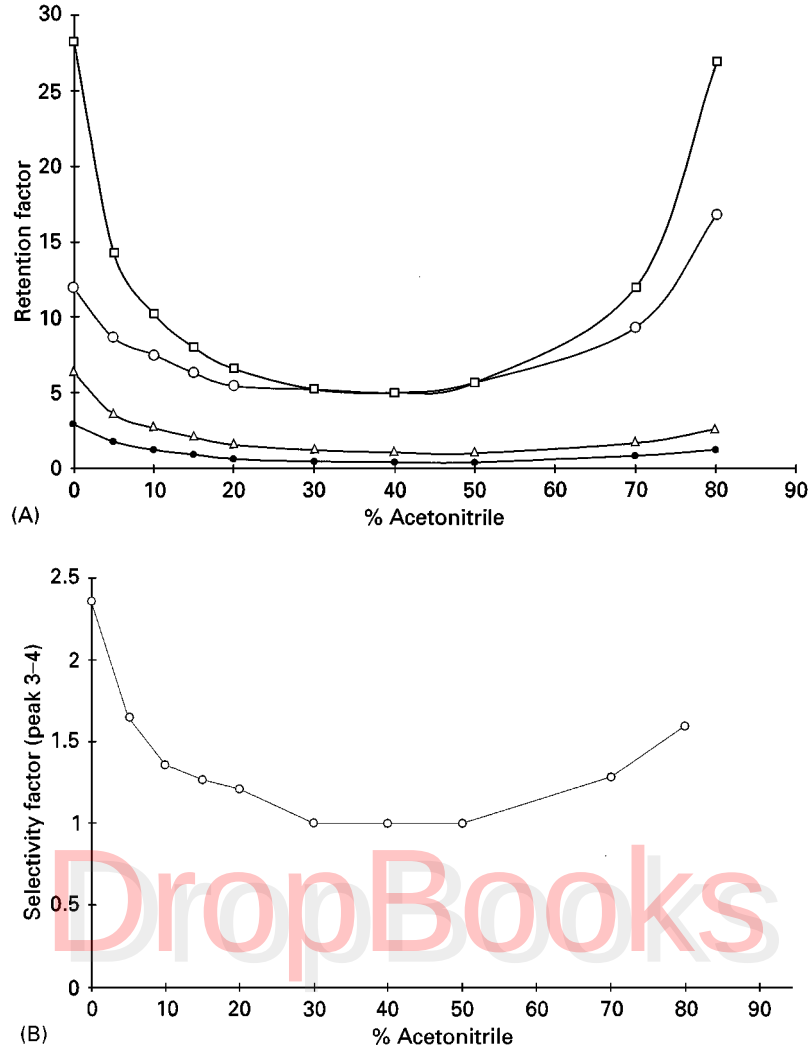


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**Figure 3** Effect of acetonitrile on (A) the retention factor; (B) enantioselectivity. Column: 250 × 4.6 mm Chiralcel AD (amylose 3,5 dimethylphenyl carbamate). Mobile phase: ethanol–acetonitrile in different ratios. Flow rate: 1 mL $min^{-1}$. Filled circles, $k'1$; triangles, $k'2$; open circles, $k'3$; squares, $k'4$.

could easily be analysed on an amylose 3,5 dimethylphenyl carbamate (Chiralpak AD) column, using ethanol–*n*-hexane in an 80 : 20 volume ratio.

A chromatogram of this separation is illustrated in **Figure 4**A.

As can be seen from **Figure 4**A, reaction with diazomethane results in different reaction products, with both nitrogen alkylated and oxygen alkylated compounds observed. Because direct analysis of this product on the cellulose- or amylose-based stationary phases, using the classical eluents, was not possible, due to the presence of the acidic NH-group in the 3,5-dioxo-1,2,4-triazin part of the molecule (which resulted in a retardation of the substance on the stationary phase), the effect of the addition of trifluoroacetic acid was examined. The result of such an experiment on a micro-LC column is shown in **Figure 4**B.

The use of an amine as tailing reducer is illustrated in the following example. A few grams of a chiral amino alcohol had to be separated in its two enantiomers. Only partial separation could be obtained on a Chiralcel OD (cellulose 3,5-dimethylphenyl carbamate) column using a mixture of *n*-hexane and 2-propanol in a 70 : 30 volume ratio. Because a severe tailing was observed, we investigated the effect on the addition of triethylamine as mobile-phase additive. A small quantity of 0.1 vol% of triethylamine improved the peak shape and the resolution. However, a much better result was observed when the triethylamine content was increased to 0.5 vol%. Thereafter, triethylamine was replaced for the same

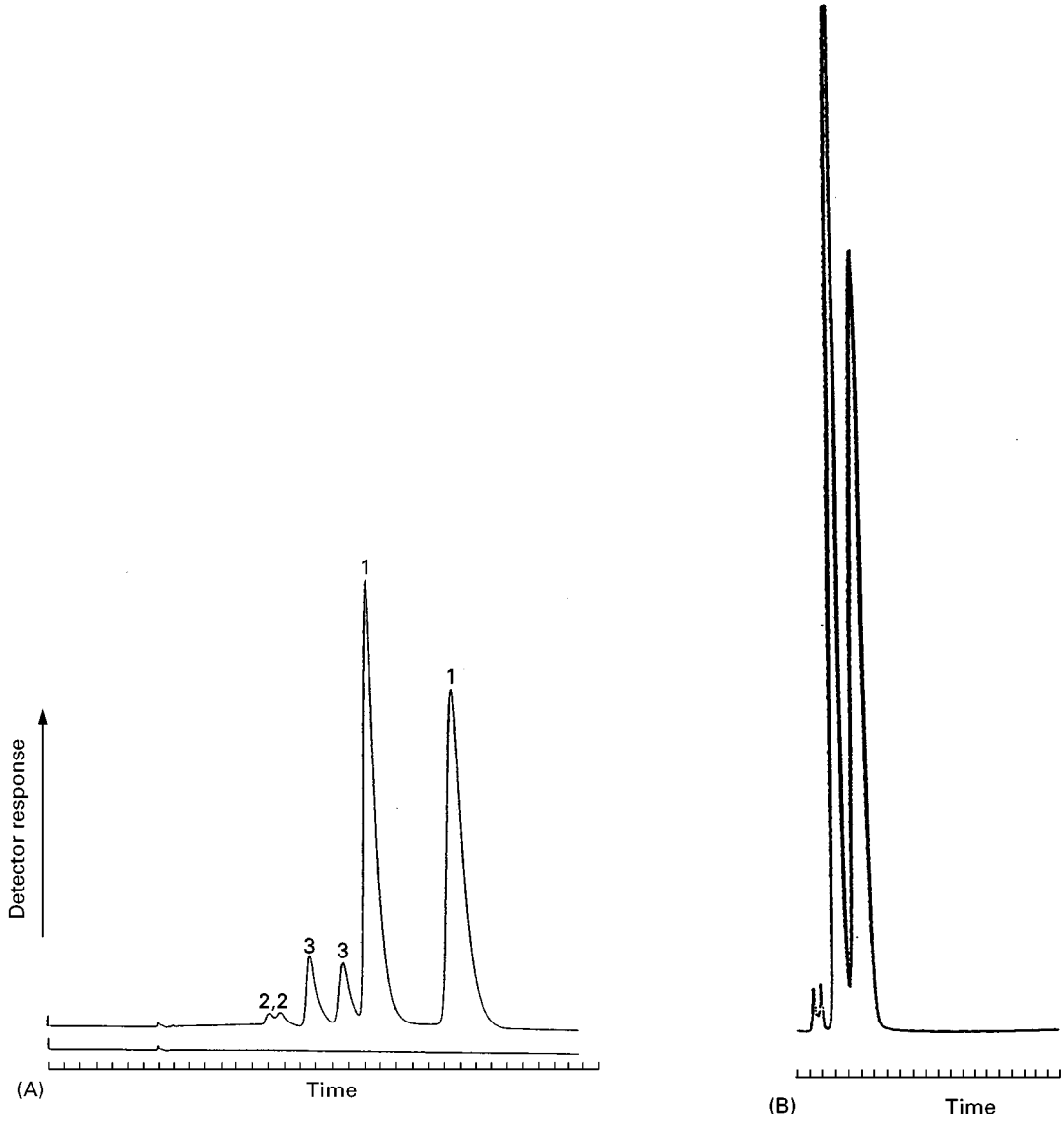


**Figure 4** (A) Separation of diclazuril after derivatization with diazomethane. Column: 250 × 4.6 mm i.d. Chiralpak AD (amylose 3,5 dimethylphenyl carbamate). Mobile phase: ethanol–*n*-hexane (80 : 20; v/v). Flow rate: 0.5 mL $min^{-1}$. Detection: UV (290 mm). Injection volume: 10 μL. Temperature: ambient. Peak 1: Enantiomers of *N*-methylated product. Peaks 2 and 3: Enantiomers of *O*-methylated product. (B) Separation of diclazuril using trifluoroacetic acid as mobile-phase additive. Column: 150 × 0.32 mm i.d. Chiralpak AD. Mobile phase: ethanol–1% trifluoroacetic acid. Flow rate: 5 μL $min^{-1}$. Detection: UV (280 nm). Injection volume: 60 nL. Temperature: ambient.

amount of diethylamine. The addition of 0.5% diethylamine resulted in the highest resolution value.

Therefore, the first experiment on the preparative column was performed with a mobile phase containing 0.5 vol% of diethylamine. The obtained result was rather poor. The enantiomers eluted as relatively broad peaks, which were only partially resolved. Because for preparative chromatographic application we prefer to work with triethylamine instead of diethylamide, the method was therefore further optimized using triethylamine as tailing reducer. To reach maximum resolution on the preparative column, the amount of 2-propanol had to be reduced from 30% to 10 vol%, while the triethylamine concentration had to be increased to 2 vol%.

Using this method, it was possible to inject 250 mg of the product. The optimized method enabled sufficient amount of the pure enantiomers to be prepared in a reasonable amount of time.

The solute structure will also clearly have a direct effect on the separation. Thus, the chiral recognition process on polysaccharide phases results from

differences in the summation of binding energies originating from:

1. hydrogen bonding
2. dipole–dipole interactions
3. charge transfer (π–π) complex formation
4. steric interactions

It is not possible to generalize which type of interaction forces plays the key role in the solute–chiral stationary phase complex formation. Hydrogen bonding certainly has a strong role in the selective chiral interaction process.

Based on the knowledge that on polysaccharide phases different mechanisms play a role in the chiral recognition process and small variations in experimental conditions or solute structure can strongly affect the enantioselectivity, it is clear that small changes in the molecular structure of a specific type of compounds that are in general well separated can often be a challenge to find an acceptable separation method for some of the members of such a product series.

The effect of small structural changes on the retention behaviour and the enantioselectivity is illustrated in the following examples.

In the first example, a few imidazole derivatives bearing an ester function on the imidazole ring were investigated on a Chiralcel OC (cellulose phenyl carbamate) as well as on a Chiralcel OD (cellulose 3,5-dimethylphenyl carbamate) column, using *n*-hexane–2-propanol as the mobile phase. On the Chiralcel OC column a mixture of *n*-hexane–2-propanol in a 90 : 10 volume ratio was used. Because with this mobile-phase composition the products were not sufficiently retained on the Chiralcel OD column, the amount of polar modifier had to be reduced to 5 vol% on this column type. On both column types the retention factor strongly decreased with increasing chain length of the eater group attached to the imidazole ring. Also steric effects seem to play a role, because on both columns the smallest retention value is measured for the 2-propyl ester.

When the resolution values are considered, a clear difference was to be observed between both column types. For the 1-propyl and 2-propyl esters, baseline resolution is obtained on both stationary phases. However, the observed differences for the ethyl and methyl ester are striking. On the Chiralcel OC column the ethyl ester is still baseline-resolved, while the methyl ester is only partially separated, whereas on the Chiralcel OD column, the methyl ester is very well separated and the ethyl ester did not display any separation at all. Certainly there is an indication that small but nevertheless influential contributions play an important role in the chiral recognition process, which of course makes it not always that easy to predict whether a new product in a series of comparable structures will be separated or not on a particular type of stationary phase.

The next example also demonstrates that it is not always easy to predict whether a product within a series will be separated on a certain type of polysaccharide phase.

Some tetramisol derivatives were investigated on three different carbamate-type phases (Chiralcel OC, Chiralcel OF and Chiralcel OD) using *n*-hexane–2-propanol in a 70 : 30 volume ratio. For the investigated compounds, the highest retention is measured on the Chiralcel OF (*p*-chlorophenyl carbamate) column, while the smallest values are observed on the Chiralcel OD column. However, on the three different column types the *meta*-substituted derivative has always the most strongly retained.

Although the products were most strongly retained on the Chiralcel OF column, not one of the 10 investigated substances was fully baseline-resolved. Two of the *ortho*-substituted compounds and the unsubstituted tetramisol were best resolved on the Chiralcel OC column, while for the *meta*- and *para*-substituted derivatives the highest resolution value was in general measured on the Chiralcel OD column.

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It is quite clear therefore from the different examples that small changes in solute structure or experimental conditions can have a strong influence on the chromatographic behaviour. This phenomenon makes it often difficult to predict whether a product will be separated on a certain type of polysaccharide phase or not. Therefore, even when a good mobile-phase composition has been found on a particular column, it is always advisable to test this solvent mixture on another column of the same type (carbamate or benzoate).

Achiral derivatization can be exploited to improve resolution as it is well known from experience that compounds bearing an ionizable group, e.g. a carboxyl, hydroxyl or amino group, are often poorly resolved on the polysaccharide type of phases. Derivatization of these functional groups to the corresponding ester, carbamate or amide derivatives frequently improves the separation.

For alcohol, for example, an esterification reaction with a *para*-substituted benzoic acid chloride has proven to be effective in solving difficult separation problems. The effect of an esterification reaction on the retention behaviour and the enantioselectivity is illustrated by means of the next examples.

The hydroxyl function of two completely different compounds was derivatized to yield the following

types of esters:

1. Benzoyl
2. *p*-Fluorine
3. *p*-Chlorine
4. *p*-Bromine
5. *p*-Trifluoromethyl
6. *p*-Methyl
7. *p*-Methoxy
8. *p*-Nitro
9. *p*-Cyano

(2–9: Benzoyl)

10. 1-Naphthoyl
11. 2-Naphthoyl
12. 9-Antracoyl

The different esters were respectively investigated on a Chiralcel OD, Chiralcel OJ, Chiralpak AD and Chiralpak AS column using pure ethanol as the eluent.

These compounds were retained more on the benzoate type of phase than on the carbamate type of stationary phase. Also, the resolution is significantly higher on the Chiralcel OJ column than on the other columns. The favourable results on the benzoate type of column inspired us to investigate the difference between ethanol and methanol as the mobile phase for this type of compound. For methanol much higher retention factors are measured than when ethanol is the eluent. In general, about twice as high resolution values are measured for methanol.

In the foregoing example, the best results for the different esters were obtained on a benzoate type of column. This is certainly not a general rule. Another alcohol [4,4-dimethoxy-1-(phenylmethyl)-3 piperidinol] was derivatized to yield a similar series of esters. This homologue series was also investigated on the four different types of polysaccharide phases, using ethanol as the eluent.

On the Chiralcel OJ column only the 2-naphthoyl derivative was partially separated. On the Chiralcel OD column only the 2-naphthoyl and the 9-anthracoyl ester were partially resolved, while on the Chiralpak AS column only the 9-anthracoyl ester showed partial resolution. However, on the Chiralpak AD column most of the products were separated. What is striking in this particular example is the partial separation of the native alcohol, while the benzoyl- and *p*-fluorobenzoyl esters do not show any separation at all.

For further synthesis applications, alcohols are frequently converted to the mesylated and tosylated derivative. When we are confronted with the preparative chromatographic separation of a racemic alcohol, we always investigate the mesylated or tosylated alcohol before carrying out other derivatization work, because we have experienced that in many cases, these compounds are easier to separate than the native alcohol. For very difficult separations we eventually had to synthesize the naphthylsulfonyl derivative.

Temperature is well known to affect chiral isolation. Thus, on chiral stationary phases, specific interaction mechanisms are involved in the separation process. Therefore, this type of phase often displays slow mass transfer characteristics.

Temperature, together with the mobile-phase velocity, certainly has to be considered as a factor that can be used to influence this mass transfer process.

In the next example, we examined the combined effects of temperature and flow velocity variations on enantioselectivity, column efficiency and resolution. The enantiomers of the investigated racemate were difficult to separate. Only partial separation could be achieved with *n*-hexane–ethanol in a 90 : 10 volume ratio on a Chiralcel OJ column, using our standard experimental conditions of flow rate and temperature. To investigate this separation problem further, the temperature was varied between 5 and 40°C and flow rates between 0.25 and 2 mL $min^{-1}$ were tested.

A good linear relationship between the logarithm of the $\alpha$ value and the temperature has been observed. The $\alpha$ value steadily increased with decreasing temperature. However, the most significant parameter to indicate the separation between two products is the resolution value. This parameter is determined by both thermodynamic and kinetic contributions.

Although two completely different measuring principles have been used, comparable patterns are observed for the two resolution values. Both figures indicate that only for temperatures below 15°C and a flow rate of 0.25 mL $min^{-1}$ can a nearly baseline separation be obtained. Although it was not possible to obtain a full baseline separation of the enantiomers, the optimized analysis method was sufficiently accurate to follow up the investigations that were performed to develop a stereospecific synthesis method.

As this example indicates, during method development and optimization experiments, the factored temperature certainly has to be investigated. Besides essential information for analytical purposes, it also gives chromatographers performing preparative chromatographic separations a good idea of whether it is possible to work at higher temperatures without losing efficiency. Due to the limited choice of possible mobile-phase compositions, the major problem one has to deal with in preparative chromatographic work on polysaccharide phases is the solubility of the product to be separated in the eluent used. Because solubility in most cases increases with increasing temperature, the ability to work at higher temperatures

often improves throughput in preparative chromatographic separations.

The experiments performed also demonstrate that the flow velocity is a parameter that can be used to optimize a separation process.

## Separation of Diastereomers

Although diastereomers are in general easy to separate under normal or reversed-phase chromatographic conditions, it often happens, especially when the chiral centres are located far away from each other, that it is not always easy to separate these compounds. When we are confronted with such a problem, we always investigate the possibilities of microcrystalline cellulose triacetate or tribenzoate and the physically coated derivatized polysaccharides. We have experienced that this approach can be a solution in many cases. An example of such a separation is described in the next example.

A racemic alcohol was derivatized with optically pure (1*S*)-camphor sulfonic acid chloride. It was not possible to separate the obtained diastereomers under normal-phase conditions on bare silica or amino-modified silica. Under the reversed-phase conditions which we generally apply in our laboratories it was not possible to separate the isomers. We did some experiments on different derivatized polysaccharide phases. On a $50 \times 4.6$ mm i.d. Chiralpak AD (amylose 3,5-dimethylphenyl carbamate) using ethanol as the eluent, the diastereomers were very well separated. Because with pure ethanol the retention factors were relatively high, the effect of the addition of acetonitrile was investigated. A few chromatograms of these experiments are depicted in **Figure 5**. Using a mixture of 95 vol% ethanol and 5 vol% acetonitrile, it was easy to isolate a large pure amount of both diastereomers.

### Reversed-Phase Applications

As mentioned before, two types of polysaccharide phases which are specifically designed for reversed-phase applications are currently on the market. This type of phase is extremely interesting for the direct injection of aqueous solutions (ionic products, plasma samples, etc.). Furthermore, the columns are useful in column-coupling techniques (for example, an octadecylsilica column followed by a polysaccharide column) to solve difficult separation problems. The manufacturer recommends on this type of column to use a mobile phase consisting of an aqueous solution of a sodium, ammonium or potassium salt in combination with methanol, ethanol or acetonitrile as the organic modifier. The choice of the cationic part of the salt seems not to have a significant effect on the separation. However, a significant difference in separation has been observed with various anions. Anions, such as $ClO_4^-$ and $PF_6^-$ in general show good separation. Also the salt concentration can strongly affect the retention time and the resolution.

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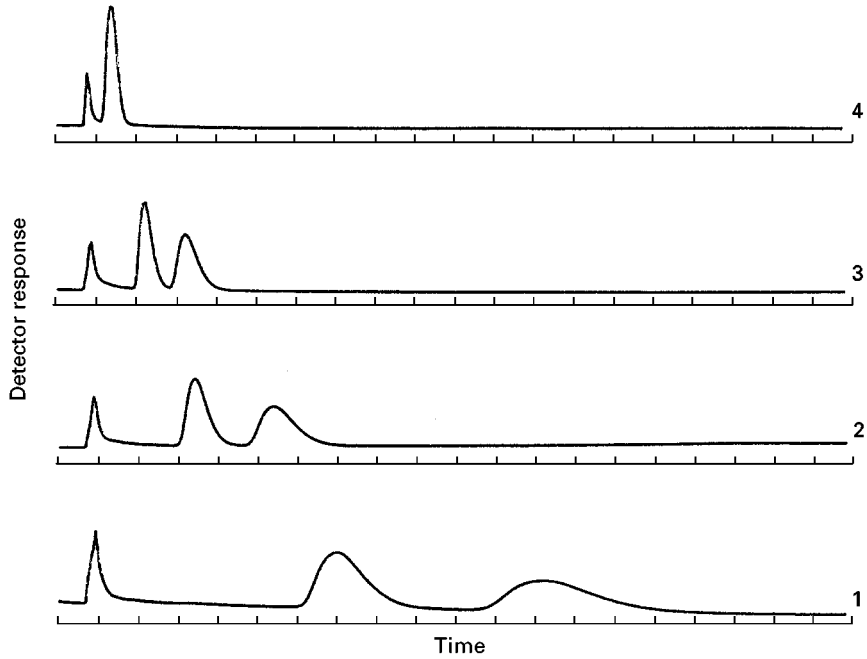


**Figure 5** Diastereomer separation. Column: $50 \times 4.6$ mm i.d. Chiralpak AD (amylose 3,5 dimethylphenyl carbamate). Flow rate: 1 mL $min^{-1}$. Mobile phase: 1, ethanol; 2, ethanol–acetonitrile (95 : 5; v/v); 3, ethanol–acetonitrile (90 : 10; v/v); 4, acetonitrile.

**Mobile-phase design and parameter optimization** Triethylamine salts are very popular as tailing reducers in reversed-phase applications. We have therefore investigated the usefulness of these salts on the polysaccharide-type phases. Experience with cyclodextrin and derivatized cyclodextrin columns taught us that a salt concentration of 50 mmol $L^{-1}$ in general is high enough to obtain good peak shapes for basic substances. Experimental work was started with this salt concentration. Afterwards, specific experiments performed to investigate the effect of the tailing reducer concentration on the chromatographic parameters confirmed that 50 mmol $L^{-1}$ was a good choice. In a concentration range between 10 and 50 mmol $L^{-1}$ the highest resolution values were always measured for the highest salt concentration. However, this concentration also resulted in the strongest retardation of the compounds.

In a first set of experiments, the difference in chromatographic behaviour of a few products under normal-phase and reversed-phase conditions was investigated on a Chiralcel OJ-R and Chiralcel OD-R column. The columns were first equilibrated with ethanol. Thereafter, a mixture of *n*-hexane–2-propanol in a 70 : 30 volume ratio was pumped through the column until equilibrium was reached and the different products were analysed. After rinsing the columns with ethanol, the column was equilibrated with a mixture consisting of 70 vol% methanol and 30 vol% of a 50 mmol $L^{-1}$ triethylamine solution in water adjusted with sulfuric acid to a pH value of 2.5.

In general the products are better retained when reversed-phase conditions are applied. It was also striking that for most of the investigated products there was a large difference in resolution values between the two modes of operation.

*Type of tailing reducer* Experiments on cyclodextrin and derivatized cyclodextrin columns have shown us that, for the analysis of basic compounds, the anionic part of the tailing reducer has a clear effect on enantioselectivity. To investigate this effect, we selected some imidazole derivatives.

To perform the experiments, a 55 mmol $L^{-1}$ solution of triethylamine in analytical grade water was prepared and 2 L portions of this solution were adjusted to a pH value of 2.5 with respectively hydrochloric, trifluoroacetic, methanesulfonic, camphorsulfonic, sulfuric and phosphoric acid. After pH adjustment the solution was further diluted to obtain a solution which contains exactly 50 mmol $L^{-1}$ triethylamine.

As eluent a mixture composed of 70 vol% of methanol and 30 vol% of the triethylamine solution was used. On the Chiralcel OJ-R and on the Chiralcel OD-R column, the retention values for phosphate or sulfate anion are markedly higher than the values measured for the other anions. The lowest values were in most cases measured for chloride as the counterion.

On both column types, the anionic part of the tailing reducer has a clear effect on enantioselectivity (**Figure 6**). In general, the best results are obtained for phosphate or sulfate as the anion, although for some products better results were obtained using another type of anion.

The Chiralcel OJ-R column is much more suitable for the separation of the investigated compounds than the Chiralcel OD-R column.

*Type of polar modifier* In experiments using a mixture of methanol and triethylamine in a 70 : 30 volume ratio, high retention times were observed for some compounds. Therefore, it was interesting to investigate the effect of the addition of acetonitrile on retention time and enantioselectivity. To perform these experiments, a mixture of 70 vol% methanol and 30 vol% triethylamine adjusted to pH 2.5 with phosphoric acid was prepared and thereafter mixed with different amounts of acetonitrile. The different chromatograms obtained during these experiments are shown in **Figure 7**.

As **Figure 7** clearly illustrates, acetonitrile strongly affects retention of the investigated compound. Although the resolution steadily decreases with increasing acetonitrile content, the $k'$ value of the second eluting peak could be reduced by a factor of 38 with a full baseline resolution of the enantiomers as a result.

Because the elution power of acetonitrile is significantly higher than for methanol, we generally prefer to start new experiments with a methanol–water-tailing reducer mixture. If the compounds of interest are too strongly retained with this eluent, methanol is systematically replaced by acetonitrile until an acceptable compromise between the retention factor and the resolution value is found.

*pH* In the analysis of ionizable compounds, pH can certainly have a strong effect on the chromatographic behaviour. We therefore investigated for the product series summarized in **Table 2** the effect of pH variations in the range between 2.5 and 4.5 on a Chiralcel OJ-R using a mobile phase composed of methanol–acetonitrile and a 50 mmol $L^{-1}$ triethylamine solution in water adjusted to the desired pH value with phosphoric acid. For one of the products investigated, the different chromatograms obtained during these experiments are depicted in **Figure 8**.

**Figure 8** shows that the retention factors steadily increase with increasing pH value, reaching

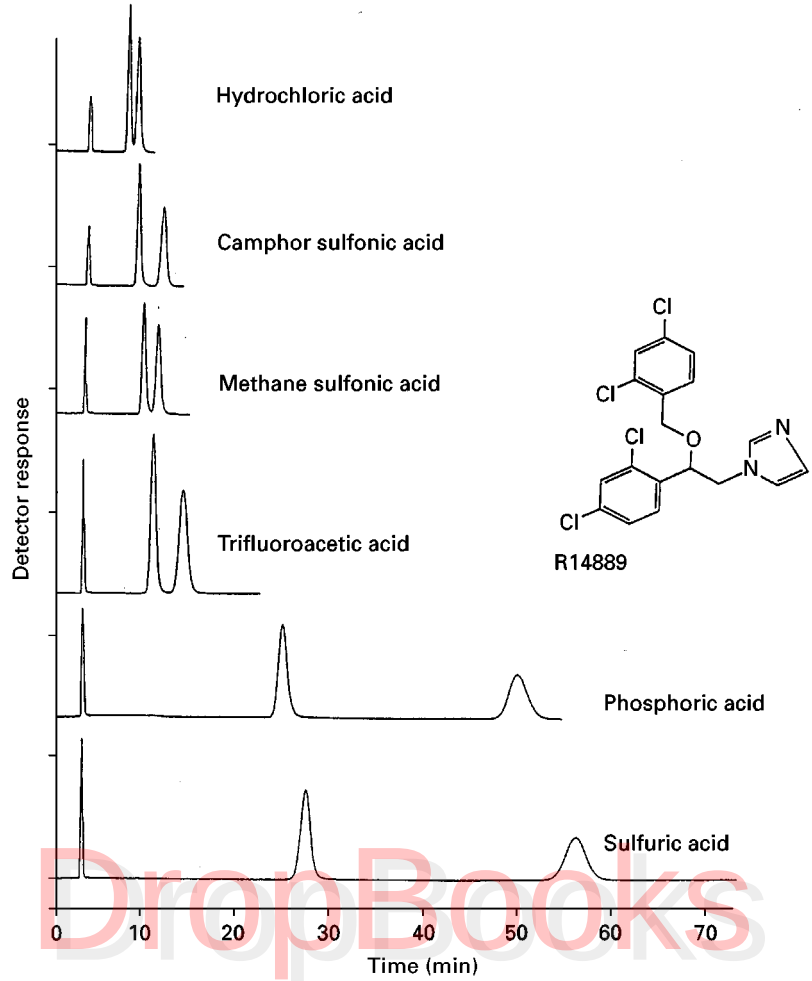


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**Figure 6** Effect of the anionic part of the tailing reducer on the resolution. Column: 250 × 4.6 mm i.d. Chiralcel OJ-R (*p*-methylbenzoyl cellulose). Flow rate: 1 mL min$^{-1}$. Mobile phase: 50 mmol L$^{-1}$ triethylamine adjusted to a pH value of 2.5 with different acids–methanol (30 : 70; v/v).

maximum at pH 4 and decreasing again above this pH value. A similar pattern was also observed for the resolution value.

The different experiments that have been performed clearly indicate that, for ionizable compounds, pH can have an important effect on the enantioselectivity, which of course makes it a parameter to be investigated thoroughly during method development and optimization.

### Chemically Bonded Polysaccharide Phases

Over the last few years, various attempts have been made to graft derivatized polysaccharides on to a silica matrix, without losing the chiral recognition properties of these materials. One of the major problems encountered was the limited amount of polysaccharide that could be chemically bonded. For the time being, only the French company Chiralsep (La Fresnaye) has a few chemically bonded polysaccharide-based columns (Chirose-bond C1 and Chirose-bond C3) on the market.

We were able to test a few experimental chemically bonded polysaccharide phases. At first we compared a chemically bonded *p*-methylbenzoyl cellulose column with the corresponding physically coated material (Chiralcel OJ) using the classical alcohol-based mobile phases. Whereas on the coated phases most of the investigated compounds were partially or completely resolved with pure ethanol as the eluent, this was not the case on the chemically bonded material. For most of the compounds investigated it was necessary to dilute ethanol with *n*-hexane to obtain an acceptable resolution value. The experiments performed certainly prove that the chiral properties of the derivatized polysaccharide were not lost during the grafting process. However, compared to the coated phases, smaller retention factors were measured on the chemically bonded material. This is

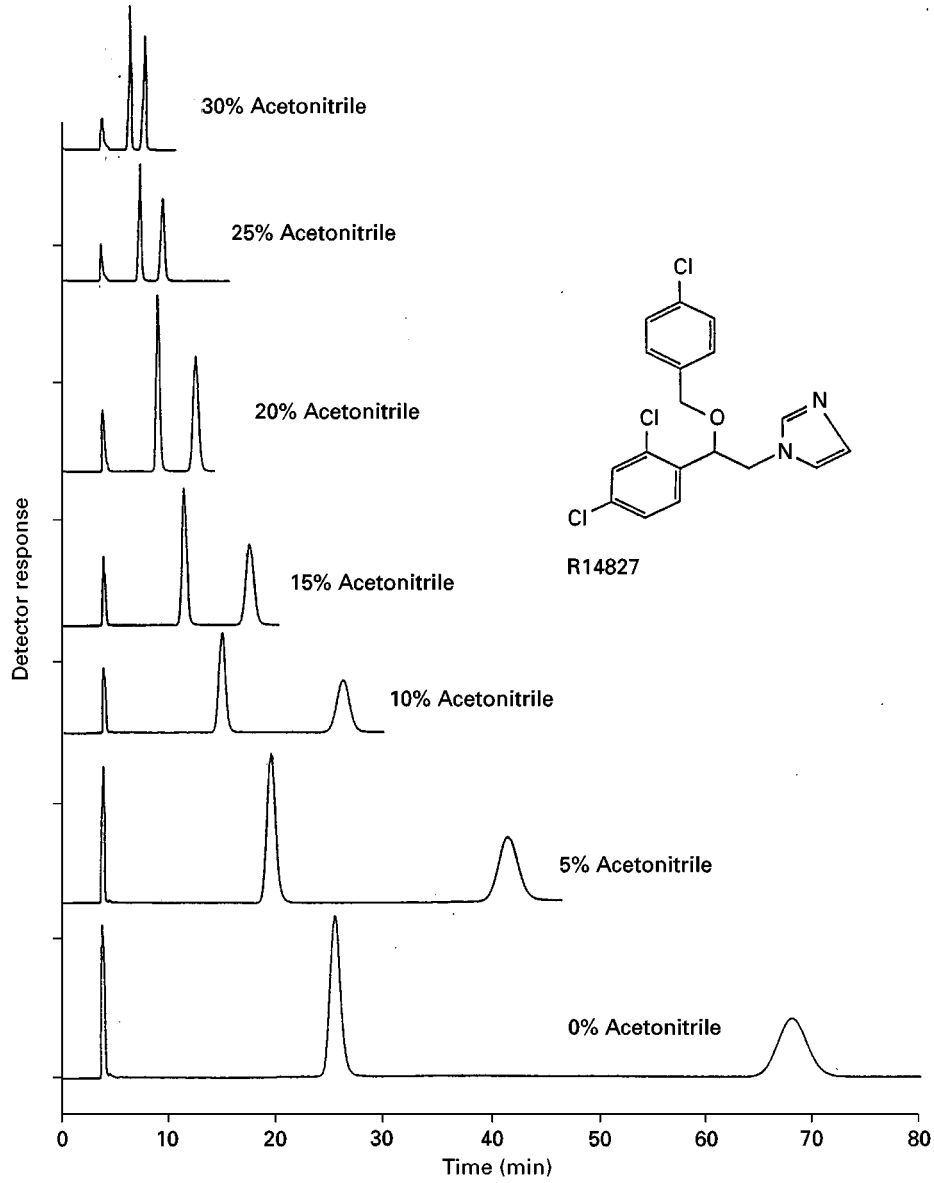


**Figure 7** Effect of type of polar modifier on chromatographic behaviour. Column: 250 × 4.6 mm i.d. Chiralcel OJ-R (*p*-methylbenzoyl cellulose). Flow rate: 1 mL min$^{-1}$. Mobile phase: 50 mmol L$^{-1}$ triethylamine adjusted to a pH value of 2.5 with phosphoric acid–methanol (30 : 70; v/v) + acetonitrile in different ratios.

possibly an indication that only a small quantity of the chiral moiety was chemically bonded on to the silica matrix. Also, some differences in enantioselective properties could be observed between both phases. Phenoperidine, for example, was not resolved with pure ethanol on the coated phase while on the chemically bonded column this product was very well separated, as illustrated in **Figure 9**.

A few experiments under reversed-phase conditions were also performed on the chemically bonded *p*-methylbenzoyl cellulose column. Methanol or ethanol in combination with a 0.5% ammonium acetate solution in water was used as the mobile phase.

For all the investigated compounds, the smallest retention and selectivity values were measured for ethanol as the organic modifier. For some products, extreme differences in resolution could be observed between the different experimental conditions applied. For an amino alcohol, the chromatograms obtained using methanol and ethanol as organic modifier are graphically compared in **Figure 10**.

Although only a limited number of experiments were performed, we can conclude that the tested chemically bonded polysaccharide phase has a high potential for reversed-phase applications.

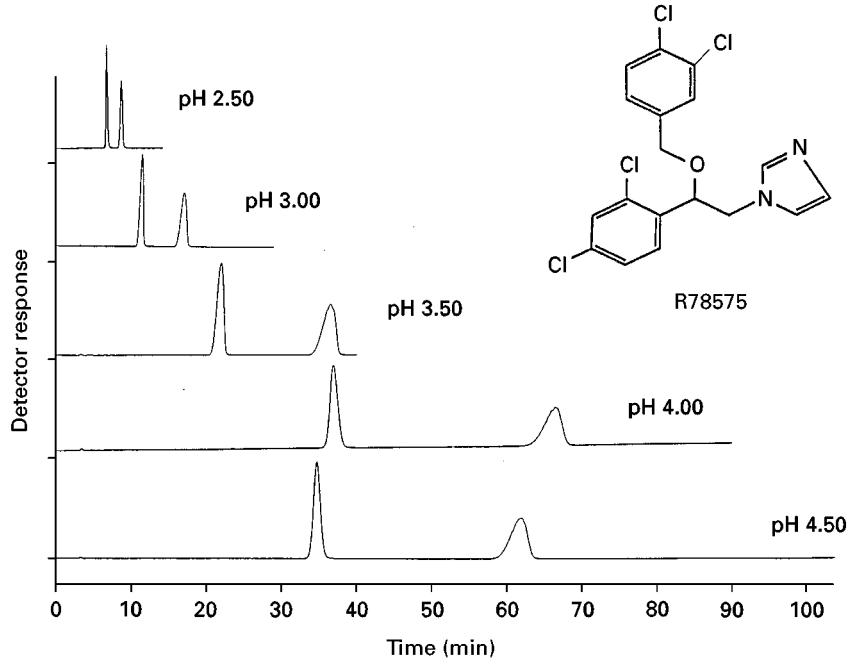


**Figure 8** Effect of pH variations on the chromatographic behaviour. Column: 250 × 4.6 mm i.d. Chiralcal OJ-R (*p*-methylbenzoyl cellulose). Flow rate: 1 mL $min^{-1}$. Mobile phase: 50 mmol $L^{-1}$ TEA adjusted to the indicated pH values with phosphoric acid–methanol–acetonitrile (30 : 40 : 30; v/v).

Besides these experiments, a series of 37 racemates belonging to different product classes were investigated on chemically bonded equivalents of respectively Chiralcel OJ, Chiralcel OD, Chiralpak AD and Chiralpak AS originating from a different source to the column used in the foregoing experiments.

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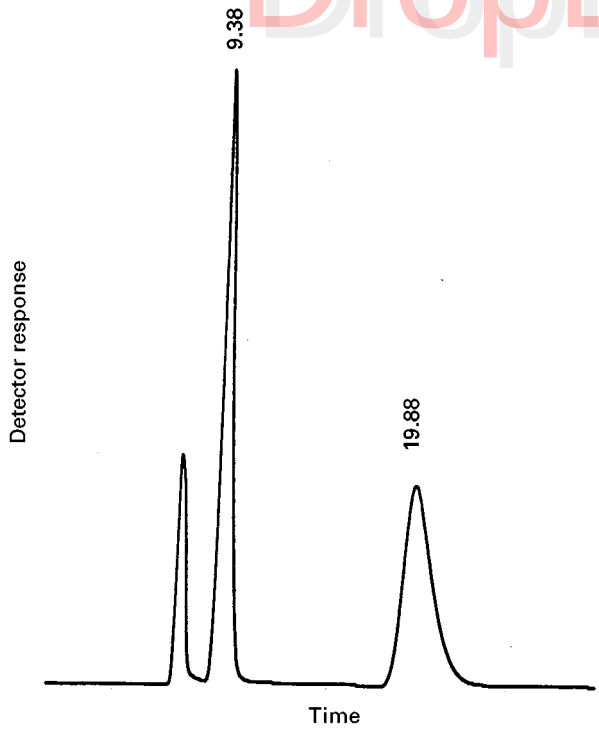


**Figure 9** Separation of phenoperidine. Column: 250 × 4.6 mm i.d. chemically bonded *p*-methyl benzoyl cellulose. Flow rate: 1 mL $min^{-1}$. Mobile phase: ethanol.

For the first tests, pure ethanol was used as the mobile phase. Using this eluent, the largest number of products were separated on the chemically bonded equivalent of Chiralcel OJ. Twenty compounds were partially or completely resolved on this column, compared to six products on the cellulose 3,5-dimethylphenyl carbamate column, 14 products on the amylose 3,5-dimethylphenyl carbamate column and eight compounds on the chemically bonded equivalent of Chiralpak AS. However, the number of products that were separated on the chemically bonded *p*-methylbenzoyl cellulose column was only 69% of the number of products resolved on the physically coated Chiralcel OJ column, using the same eluent. Because for the same eluent composition similar retention factors were measured on the chemically bonded columns and their physically coated equivalents, we may conclude that the amount of cellulose or amylose grafted on to the silica matrix has to be the same order of magnitude as the amount present on the physically coated phases.

Other solvents were also used on these columns. After extensive use of dichloromethane, which normally dissolves cellulose and amylose derivatives, the properties of the columns were tested again and compared with the results obtained on a fresh column. Practically no difference in retention or resolution

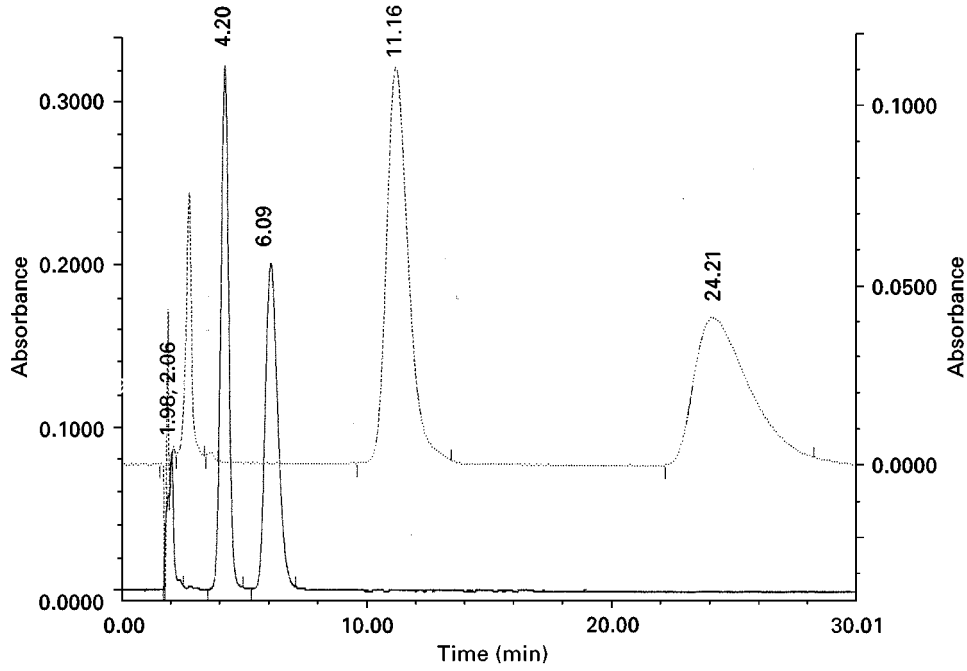


**Figure 10** Separation of an amino alcohol under reversed-phase conditions. Column: 250 × 4.6 mm i.d. chemically bonded *p*-methylbenzoyl cellulose. Flow rate: 1 mL $min^{-1}$. Mobile phase: continuous line, 0.5% ammoniumm acetate in HPLC-grade water–ethanol (50 : 50; v/v); dotted line, 0.5% ammonium acetate in HPLC-grade water–methanol (50 : 50; v/v).

values could be observed before and after the use of dichloromethane. This is an indication that the polysaccharide derivative was perfectly bonded on to the silica matrix.

Some of our products were also investigated under normal-phase conditions on the Chirose-bond C1 column of Chiralsep. Most compounds eluted as relatively broad peaks with a severe tailing. From the 22 investigated compounds, only three products were fully baseline-resolved. The addition of a small amount of triethylamine did not solve the tailing problem. For only one of the investigated compounds was a very good resolution observed on the Chirosebond C1. The results of these experiments are summarized in **Table 3**.

The ability to use dichloromethane for this compound is interesting for preparative chromatographic purposes, because the solubility of this product in the commonly used solvents (alcohol, or alcohol–*n*-hexane mixtures) is rather poor.

**Table 3** Use of dichloromethane on chemically bonded cellulose derivative

| *Mobile-phase composition* | *k′2* | *α* | *Resolution* |
|---|---|---|---|
| *n*-Hexane–2-propanol (45 : 55; v/v) | 3.93 | 2.15 | 5.88 |
| *n*-Hexane–2-propanol–dichloromethane (40 : 50 : 10; v/v) | 3.14 | 1.97 | 4.63 |

Some of the chemically bonded polysaccharide phases which have been tested certainly have a high potential. The possibility of using a broader pallet of solvents or solvent combinations especially widens for preparative chromatographic work the field of application.

## Conclusions

Different types of derivatized cellulose and amylose stationary phases are nowadays commercially available. With a limited number of these phases it is possible to separate a broad variety of products. Furthermore, these phases are also extremely useful for preparative chromatographic applications.

Although differences in chromatographic behaviour between the tested chemically bonded phases and their physically coated equivalents have been observed, we might expect that, after further optimization of the grafting process, these phases will certainly enlarge the field of application of the derivatized cellulose and amylose materials.

*See also:* **II / Chromatography: Liquid:** Chiral Separations in Liquid Chromatography: Mechanisms. **III / Chiral Separations:** Amino Acids and Derivatives; Capillary Electrophoresis; Chiral Derivatization; Countercurrent Chromatography; Crystallization; Cyclodextrins and Other Inclusion Complexation Approaches; Gas Chromatography;

Ion-pair Chromatography; Ligand Exchange Chromatography; Liquid Chromatography; Molecular Imprints as Stationary Phases; Protein Stationary Phases; Supercritical Fluid Chromatography; Synthetic Multiple Interaction ('Pirkle') Stationary Phases; Thin-Layer (Planar) Chromatography.

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# Chiral Derivatization

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## Introduction

### The Importance of Enantiomeric Separations

The separation of enantiomers of chiral compounds by chromatographic methods and related techniques is one of the important tasks in modern analytical chemistry, especially in the analysis of compounds of biological and pharmaceutical interest. However, analysis of this kind is also required in food analysis and the analysis of pesticides, flavours and fragrances. The reasons for this are as follows:

- As a consequence of the existing or potential differences between the biological–pharmacological activities of the antipodes of racemic drugs, analytical methods are required for their simultaneous determination in biological samples, thus enabling one to follow the fate of the enantiomers of the administered drug (candidate) in the animal or human organism.
- Asymmetrical syntheses are in the focus of interest in various fields of organic chemistry especially in the synthesis of drugs administered as the pure enantiomer. In these cases and also if the preparation of the enantiomers is carried out by classical resolution techniques, analytical methods are necessary for the determination of their enantiomeric purity.

### Possibilities for Enantiomeric Separations

Since in achiral environment the physicochemical properties of the antipodes of racemates are identical, their separation is not possible if the generally used, achiral separation systems – ordinary high-performance liquid chromatography (HPLC) and gas chromatography (GC) columns, thin-layer chromatography (TLC) plates, capillary electrophoresis (CE) capillaries, etc., with ordinary mobile phases – are used. The main possibilities for the separation of enantiomers are:

- Transformation of the enantiomers to covalently bonded diastereomeric derivatives by reacting them with homochiral derivatizing reagents prior to their chromatographic separation using achiral stationary phases or and mobile phases. The detailed description of this general method, which is often referred to as an indirect method, is described here.
- Incorporation of the chiral reagent in the mobile phase for the dynamic formation of diastereomeric adducts, ion pairs or complexes with the enantiomers to be separated during the chromatographic run. In this case also, achiral stationary phases are used.
- Separation of the enantiomers on chiral stationary phases (HPLC, GC and TLC). Although in principle this general method does not require derivatization, the separation can be improved in many cases by modifying the enantiomers using precolumn achiral derivatization. These aspects are also briefly discussed here.

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## Covalent Chiral Derivatization of Enantiomers and Separation of the Diastereomeric Derivatives on Achiral Columns

### Introductory Remarks: the Role of Covalent Chiral Derivatization in Enantiomeric Separations

The derivatization of enantiomers using homochiral reagents to form their diastereomeric derivatives